EFFECT OF BMP SIGNALS IN ANTERIOR AND POSTERIOR NEURAL MODELS ON REGULATING THE FGF PATHWAY

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ABSTRACT

Objective*: To analyse the effect of the bone morphogenetic protein (BMP) signal on regulating the fibroblast growth factor (FGF) pathway in anterior and posterior neural models, and to investigate the specific mechanism of how FGF action is eliminated by the neural plate in the early development of vertebrates.*

Methods: *The levels of the phosphorylated extracellular signal-associated kinase (p-ERK) in the dorsal ectoderm along the anterior-posterior (A-P) axis of the ganglion stage embryos were measured. FGF signalling activity was detected to determine the hierarchical activity model of the BMP and FGF signals. We also detected whether BMP could inhibit the FGF signal and analysed whether the signal was involved in the anterior nerve induction by controlling FGF signal transduction using SU5402. Also, we investigated whether BMP4 can inhibit the expression of FGF8-induced Flrt3.*

Results: *The findings of this study indicated that BMP signals can act as an antagonist of FGF signalling in the A-P neural model in Xenopus laevis embryos. During the ganglion phase, BMP signals were up-regulated in the anterior nerve plate, showing a hierarchical model along the A-P axis. Inhibiting the late BMP signal transduction after the formation of intermediate gastrula embryos eliminated the expression of the anterior nerve markers. We found that BMP signals interfered with FGFs-induced ERK phosphorylation and neuro-caudalisation. This inhibition of BMP signals would involve controlling the expression of Flrt3, a positive regulator of FGF signalling. In addition, we found that Flrt3 got and lost functions restrained and amplified the expression of the forebrain marker gene, respectively.*

Conclusions: *These findings suggest that BMP signals can downregulate the anterior nerve formation by limiting Flrt3 expression to downregulate the FGF pathway, revealing the stage-specific function of the BMP signal pathway and its new role in neural development.*

Keywords: *BMP signals, FGF pathway, anterior and posterior nerves.*

DOI: 10.19193/0393-6384_2020_6_562

Received *November 30, 2019;* ***Accepted*** *January 20, 2020*

Introduction

The formation of the vertebrate nervous system is caused by neural induction, which distinguishes the neural plate from non-neural (epidermal) ectoderm. The neural model then induces the specific gene expression along the anterior-posterior (A-P) axis in the neuroectoderm. In the two-signal model proposed by Nieuwkoop, neural induction initially produces the anterior neural tissue (forebrain), which is then guided to be more posterior neural tissue (midbrain, hindbrain, and spinal cord) by tail signals[(1)]. Some studies in amphibians have shown that limiting bone morphogenetic protein (BMP) signals is sufficient to induce neural tissue in ectodermal tissue. Therefore, neural induction requires secretion signals, especially BMP antagonists such as noggin and chordin, stemming from the back mesoderm or the embryo of Spemann tissue region[(2)]. In addition, studies on chicken and frog embryos have shown that fibroblast growth factor (FGF) signalling is involved in neural induction processes.

The tailing factors involved in the A-P pattern inducing neural tissue include Wnt, FGF, Nodal and

retinoic acid. Spemann tissue also regulates the A-P neural model by secreting inhibitors of tailing factors such as Dickkopf-1, Lefty, Frzb-1 and Cerberus. These inhibitors play a key role in the formation of the anterior neural ectoderm by limiting the effects of Wnt, Nodal, and BMP signalling in a non-cellular autonomous manner. FGF ligands, such as FGF4 and FGF8, are expressed in the mesoderm and posterior neuroectoderm, which are also considered to be a key factor in the A-P structure of neural tissue. Shisa has been reported to attenuate FGF signalling in the anterior neural ectoderm, which is an endoplasmic reticulum (ER) protein that inhibits the transportation of the FGF receptor toward to the cell surface. However, the low-vel mechanisms of FGF signalling transduction caused by the anterior neural ectoderm have not been fully elucidated[3].

Some growth processes appear to be regulated by cross-linking between BMP and FGF signalling pathways. FGF may promote neural induction through MAPK-mediated phosphorylation of the BMP-specific Smad1 linker region, which results in the retention of the Smad1 cytoplasm and the inhibition of BMP signalling. The inhibition of BMP signals has been shown to initiate neural induction by activating FGF4 expression. In addition, BMP inhibiting FGF signalling plays a vital role in limb structuring and cardiac differentiation. From these studies, it was concluded that both FGF and BMP-mediated pathways should be maintained at appropriate levels for normal development and that maintaining a balance between FGF and BMP signalling seems to require mutual regulatory mechanisms. In this study, we investigated the resistance of BMP signals to an FGF neural signal model. After gastrula embryo formation, BMP signals are activated in the anterior neural ectoderm of the early embryo of Xenopus laevis. This late activation of BMP signals is essential for keeping the anterior nerve function. The activity of BMP signals is associated with the downregulation of FGF signalling, achieved by inhibiting the expression of Flrt3 (the posterior-anterior regulator). Therefore, we propose that BMP signals can act as a novel inhibitor of the FGF tailing pathway in the A-P structuring of neural tissues.

Materials and methods

Embryo preparation and chemical treatment

In vitro fertilization, microinjection and embryo culture were performed as previously described (4). The developmental stage of the embryo was determined according to the normal development table of Nieuwkoop and Faber[5]. To induce the human glucocorticoid receptor ligand-binding domain (hGR)-fusion construct, dexamethasone (DEX; 10 μM, Sigma) was added at the appropriate time. SU5402 (20 μM, Calbiochem) was used at specified stages to block FGF signalling.

Plasmid construction and morpholino oligonucleotides

The full-length Flrt3 of X. laevis was amplified using PCR and the BamHI / XhoI site of the pCS2 + vector was inserted. Antisense morpholino oligonucleotides (MO) were purchased from Gene Tools. To completely deplete Flrt3, equal amounts of Flrt3 MO-a and Flrt3 MO-b were mixed and microinjected. The control MO was a standard morpholino oligonucleotide.

Immunostaining and western blot

The embryos were collected and fixed in MEM-FA for 1 h at room temperature and then incubated in PBS + 0.1% Tween20 lysine for 10 min. The embryos were washed several times in polybutylene terephthalate (PBT) (phosphate-buffered saline (PBS), 2 mg/ml ovine serum albumin (BSA), 0.1% Triton X-100), and stored in PBT for up to 24 h at 4°C before immunostaining. Immunostaining: the embryos were sealed in PBT containing 10% normal goat serum (NGS), incubated in anti-pSmad1/5/8 antibody diluted 1:20 in 10% NGS overnight at 4°C, washed in PBT six times, and then stained for 15–20 min using ImmunoPure Metal-enhanced DAB Kit (Pierce) according to the manufacturer's instructions. p44/42 MAPK (Erk1/2) Smad1/5/8 (1:1000, cell signal) was phosphorylated using p44/42 MAPK (Erk1/2) following the standard protocol. Smad1 (1:1000, Santa Cruz) and anti-muscle according to standard protocol. Smad1 (1:1000, Santa Cruz) and anti-actin (1:1000, Santa Cruz) were subjected to Western blotting for 15 min and then incubated with HRP-conjugated secondary antibody (1:250).

RT-PCR pedigree tracing, in situ hybridization and RT-PCR

B-galactosidase mRNA was injected, and its activity was demonstrated with Red-Gal substrate (Sigma). Bulk in situ hybridization was conducted. Antisense in situ RNA probes was synthesized in vitro using digoxigenin-labelled nucleotides. For RT-PCR analysis, total RNA was extracted from whole embryos and tissue explants using TRI Reagent, and

RNA was transcribed using M-MLV reverse transcriptase at 37°C for 1 h. The PCR products were analysed on a 2% agarose gel. The number of PCR cycles per primer set was determined empirically to maintain the amplification in the linear range.

Results

Hierarchical activity model of BMP and FGF signals

In that FGF signal regulation induces the A-P model of neural tissue, we desire to ascertain whether the level of FGF signalling transduction is directly related to its function in the neural model. Therefore, we measured the levels of the phosphorylated extracellular signal-associated kinase (p-ERK) and FGF signalling activity along the dorsal ectoderm of the ganglion stage embryos. Consistent with its role as a posterior factor in the neural model, FGF signalling activity in the tail was usually higher than that in the medullary region of the embryo (Figure 1A). We also tested the pattern of BMP signal transduction activity in the same tissue. In contrast to p-ERK levels, phosphorylated Smad1 (p-Smad1) produced by BMP activation was more prominent in the anterior region, which appears to include the mucous glands and forebrain, rather than the posterior region of the mid- and hindbrain and the spinal cord (Figure.1A). This hierarchical p-Smad1 model was also noted in neuro-embryonic whole embryos (Figure 1B). These models of BMP and FGF signalling activity prompted us to hypothesize that BMP signals can downregulate FGF signalling in the anterior and posterior neural models.

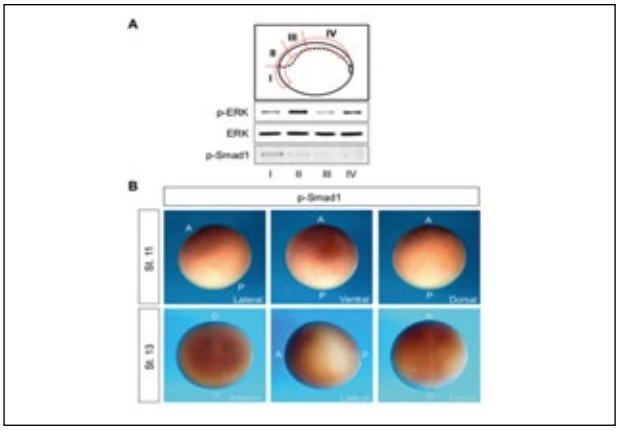

Figure 1: BMP signals form a hierarchical pattern along the anterior and posterior axes of the nerve.
(A) Western blot analysis shows the activity of FGF and BMP signals in the dorsal neuroectoderm, which is subdivided into four parts along the A-P axis of the neuronal embryo (I, II, III, and IV). (B) Immunohistochemistry of phosphorylated Smad1 on whole embryos at stages 11 and 13. As shown in the Figure, the embryos were observed, A, anterior; P, posterior; D, dorsal; V, abdomen.

BMP weakens FGF signalling transduction

In embryonic cells of X. laevis, the basal level of p-ERK was too low to be effectively detected (Figure 2A), possibly due to the low FGF signalling activity mentioned earlier. BMP inhibitor expression slightly increased its level (Figure 2A), suggesting that a low basal level of p-ERK was also caused by a high level of BMP signalling in animal cells. HEK 293T cells had relatively high level of p-ERK, which was downregulated by co-expressing BMP2 or BMP4 (Figure 2B) In addition, FGF8 or eFGF-enhanced p-ERK levels were increased by decreasing BMP expression, respectively (Figure 2C, D). Co-injecting BMP4 could inhibit the ectopic expression of FGF8-induced posterior neural marker HoxB9 (Figure 2E).

However, BMP4 could not effectively interfere with FGF8-induced ERK phosphorylation in the presence of cyclohexanimide, a protein synthesis inhibitor (Figure 2F), suggesting that the mechanism by which BMP inhibits FGF signalling involves the negative regulators transcribing and inducing the FGF pathway. Interestingly, the inhibition of BMP4 on FGF8-induced ERK phosphorylation reached a bottleneck, and there was still a certain level of FGF activity even with the BMP4 increase (Figure 1G). Since ERK phosphorylation can be triggered by factors other than FGF (6), we investigated whether BMP4 can inhibit ERK phosphorylation in response to insulin-like growth factor-1 (IGF-1) and found that BMP4 didn't make difference (Figure 2H). Moreover, BMP4 signals could not affect the stabilization of β-catenin induced by Wnt signalling in another tailing pathway (Figure 2I). In sum, these results indicated that BMP signals downregulated FGF signalling by inducing specific inhibitors of this pathway.

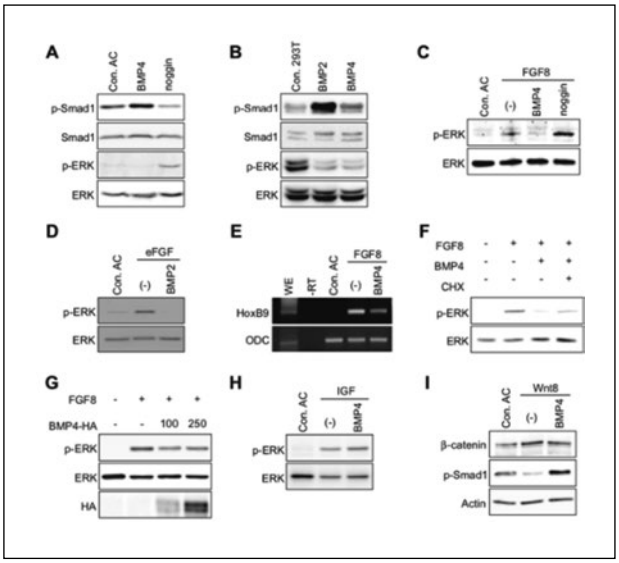

Figure 2: BMP weakens FGF signalling transduction.

Late activation of BMP signals is essential for anterior nerve development

Since BMP inhibition upregulated FGF signalling (Figures 2A and C), we investigated whether this FGF signalling enhanced by BMP inhibition might be involved in the anterior neural induction. As expected, SU5402-controlled FGF signalling can impair the noggin or chordin-induced expression of the anterior neural marker Otx2 and pan-nerve marker NCAM (Figure 3A).

These results revealed that inhibiting BMP signals might play a role in limiting FGF signalling. Given that FGF signalling directly induces several neural markers in a dose-dependent manner, higher doses triggered more tail nerve genes (Figure 3B). The anterior nerve tissue was induced when the BMP inhibition lost validation, and FGF could be at a low level. BMP signals were activated in the anterior neural plate after gastrula embryo formation (Figure 1B) and acted as a specific inhibitor of the FGF pathway, as shown above. Therefore, we hypothesized that activating but not limiting BMP can induce anterior nerve tissues in animal cells. A high dose of FGF8 only induced the expression of the posterior neural markers HoxB9 and Krox20, but not the expression of the anterior neural marker Otx2. BMP signals activated by Smad1-GR could also be activated in the early and late stages. Meanwhile, this gene expression model could be reversed around the middle embryo stage (Figure 3C). These data suggested that precursor neural tissue can be induced by limiting or activating BMP signals, which is dependent on the level of FGF signals in the cells.

We further investigated whether BMP activation is essential for in vivo neurodevelopment and found that the expression of DSmad7tevGR and TEV2GR in animal cells covers effectively inhibited Smad1 phosphorylation in a DEX-dependent manner (Figure 3D). Similarly, embryos co-injected with DSmad7tevGR / TEV2GR RNA and treated with stage 3 to 15 DEX presented amplified expression of the mucosal glands and forebrain markers Otx2 and the forebrain marker Bf1.

In contrast, the expression of these anterior neural markers appeared to be significantly blocked in embryonic stages injected with the same RNA and treated with 10.5 to 15 DEX (Otx2, 73.6%, n = 38; Bf1, 79.4%, n = 39; Figure 3E). In conclusion, to achieve the optimal level of FGF signalling in normal anterior neurogenesis, BMP signals should be suppressed and activated before and after the midgut stage, respectively.

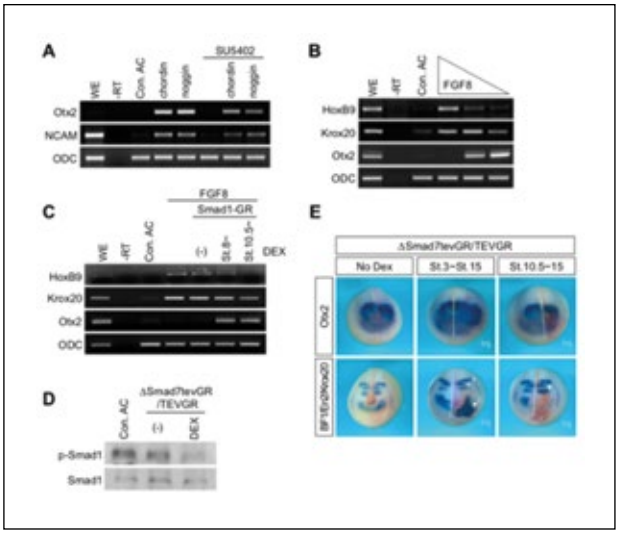

Figure 3: Activating BMP is necessary to maintain the anterior nerve.

BMP antagonizes FGF signalling by inhibiting Flrt3 expression

According to Figure 4A, it is known that Flog3 expression might be strongly induced by noggin or dominant-negative BMP receptor tBR (truncated BMP receptor)-mediated ectoderm explant BMP. BMP4 also could inhibit FGF8-induced Flrt3 expression, while Flrt2 (tight isoform of Flrt3) expression was not affected by BMP inhibition or activation (Figure 4A and B), indicating that BMP plays a specific role in Flrt3 expression.

Knockout of the Flrt3 gene mediated by antisense morpholino oligonucleotide (MO) inhibited FGF8-induced ERK phosphorylation (Figure 4C). In addition, the deletion of Flrt3 prevented the ERK phosphorylation induced by the noggin protein (Figure 4D), and its co-injection rescued the inhibitory effect of BMP4-induced ERK phosphorylation (Figure 4E), thus confirming that BMP inhibits FGF by limiting Flrt3 expression signalling. We investigated whether the level of Flrt3 expression is related to the A-P neural model. As shown in Figure 4F-I, Flrt3 MO injected into the anterior nerve tissue extended the expression of the anterior nerve markers Otx2 and Bf1 (Otx2 is 95%, n = 40; Bf1 is 72%, n = 39), and when Flrt3 was injected into the same tissue, Flrt3 RNA inhibited their expression (Otx2 was 84%, n = 38; Bf1, 78%, n = 41). Although the knockout of Flrt3 reduced the expression of En2 and Krox20 markers, its overexpression had the opposite effect, namely having a lateral and forward extension effect on Krox20 (Figure 4H and I). These results indicate that the expression level of Flrt3 plays a key role in the formation of posterior nerves.

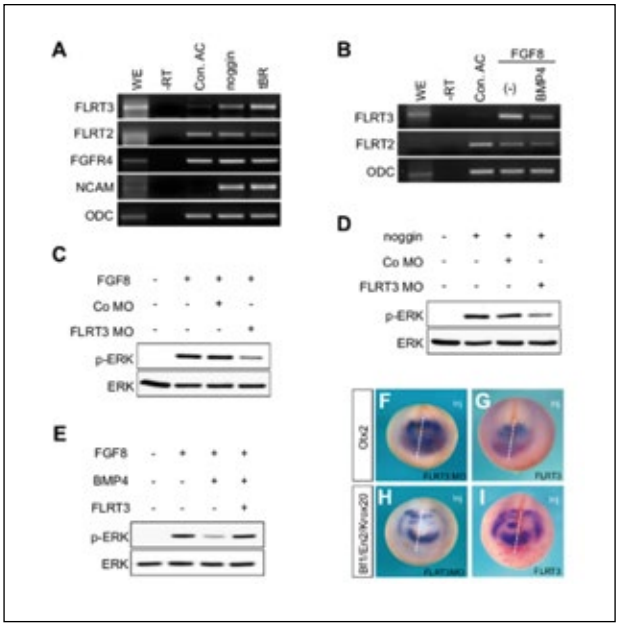

Figure 4: BMP signals inhibited Flrt3 expression to downregulate FGF pathway.

(A–E) Four-cell stage embryos were injected into the animal polar regions, as shown by noggin (10 pg), tBR (100 pg), FGF8 (1 ng), BMP4 (200 pg), Flrt3 (200 pg), and Flrt3 MO (12.5 ng) and Co MO (12.5 ng), then the animal explants were excised at stage 8 and cultured to stage 12 for RT-PCR (A, B) or Western blot (CE) analysis. Figure 4. BMP signals inhibited Flrt3 expression to downregulate the FGF pathway. (A–E) The four-cell stage embryos were injected into the polar regions of the animal, as shown by noggin (10 pg), tBR (100 pg), FGF8 (1 ng), BMP4 (200 pg), Flrt3 (200 pg), Flrt3 MO (12.5 ng) and Co MO (12.5 ng), then the animal cap explants were excised at stage 8 and cultured to stage 12 for RT-PCR (A, B) or Western blot (CE) analysis. (F-1) Four-cell stage embryos were injected into the Flrt3 (200 μg) or Flrt3 MO (10 ng) animals, cultured to stage 15, and then Otx2, Bf1, En2, and Krox20 were performed in situ hybridization.

Discussion

In this study, we find that BMP signals are required for the anterior neural development of X. laevis. The anterior nerve induction has been shown to occur in the pre-gastrula stage[7-8]. Inhibiting BMP signals only after the mid-gastrula stage reduces the expression of the anterior nerve markers, suggesting that this late BMP signal is critical for maintaining the anterior nerve characteristics. The mechanism of BMP signal transduction involves that it weakens the posterior FGF signalling by inhibiting Flrt3 expression. To support this, the knockout of Flrt3 suppressed the upregulation of ERK phosphorylated noggin, and its co-expression restored the FMP8-induced BMP inhibition of ERK phosphorylation. Moreover, Flrt3 depleted embryos showed enlarged expression of anterior neural markers, but relatively reduced expression of posterior neural markers such as En2 and Krox20, thus confirming that Flrt3 expression levels are correlated with the A-P neural model. It has been demonstrated that FGF signalling inhibits BMP signal transduction through MAPK-mediated Smad1 phosphorylation. FGF signal transduction can also induce Flrt3 expression to produce positive feedback. In short, these results indicate that the antagonism between BMP and FGF signal transduction plays a key role in precisely controlling the A-P neural model.

Antagonistic interactions between the BMP and FGF pathways can ensure their balance, which is critical for dividing spatial boundaries or developmental thresholds. Inhibiting FGF signalling through the BMP pathway is necessary for ectoderm cells to maintain epidermal and anterior neural features. This is true even in the presence of an increased BMP signal increase. The inhibition of BMP on FGF signalling has been shown to reach a plateau (Figure 2G), suggesting that FGF signalling may block the BMP pathway. For example, the activity of the suppressed former is below the threshold via the inhibitory phosphorylation of Smad1. This adverse effect of FGF signalling may be a requirement of the anterior nerve induction of the BMP antagonist and the posterior nerve model after gastrula embryo formation[9]. Future experiments are necessary to further study whether the inhibition of BMP signals on the Flrt3 / FGF pathway may occur in developments beyond neural models and identify other target genes of BMP signals. In addition, whether these genes can mediate the cross-reaction between BMP and other signalling pathways will be investigated.

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